IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appellants	:	Ivan KING and Li-Mou ZHENG
U.S. Serial No.	:	10/738,423
Confirmation No.	:	8783
Filed	:	December 16, 2003
Art Unit	:	1633
Examiner	:	Qian Janice Li
For	:	COMPOSITIONS AND METHODS FOR TUMOR-TARGETED DELIVERY OF EFFECTOR MOLECULES

Law Offices of Albert Wai-Kit Chan, PLLC
World Plaza, Suite 604
141-07 20th Avenue
Whitestone, New York 11357

January 9, 2009

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir/Madam:

RESPONSE TO NOTIFICATION OF NON-COMPLIANT APPEAL BRIEF

A Notification of Non-Compliant Appeal Brief (37 CFR 41.37) was mailed on December 31, 2008. To avoid dismissal of the appeal, reply to the Notifications must be submitted within the longest of one month or thirty days from the mailing date of the Notifications, i.e. by January 31, 2009. Accordingly, this response is being timely filed.

In the Notification of Non-Compliant Appeal Brief (37 CFR 41.37), it is stated that the Appeal Brief filed October 9, 2008 was defective for not containing copies of evidence submitted under 37 CFR

1.130, 1.131, or 1.132 or of any other evidence entered by the examiner and relied upon by appellant in the appeal, along with a statement setting forth where in the record that evidence was entered by the examiner, as an appendix thereto (37 CFR 41.37(c)(1)(ix)).

Applicants submit that the Appeal Brief has been amended to include copies of evidence as an appendix as required by 37 CFR 41.37(c)(1)(ix). Accordingly, Applicants submit that the appeal brief submitted herein has been amended to fully comply with 37 CFR 41.37.

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

The real party in interest is Vion Pharmaceuticals, Inc. by virtue of an assignment recorded on October 16, 2001, at Reel/Frame 012277/0251.

II. RELATED APPEALS AND INTERFERENCES

No appeals or interferences have been filed to the knowledge of the Appellants, Applicants' undersigned attorney, or the assignee that are related to or would be affected by a decision by the Board of Patent Appeals and Interferences in this pending appeal.

III. STATUS OF CLAIMS

Claims 1-112, and 114 have been canceled. Claims 113, 115-117, 119-124 were rejected; claims 118 and 125 were withdrawn from consideration. Consequently, claims 113, 115-117, 119-124 are currently appealed.

IV. STATUS OF AMENDMENTS

A Final Office Action was issued on December 20, 2007. No further amendments have been filed subsequent to the amendment filed October 22, 2007; therefore, no amendments were filed subsequent to final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 113 is drawn to a method of inhibiting the growth of a solid tumor cancer, comprising administering to a subject an effective amount of cytoxan or cisplatin and an effective amount of a pharmaceutical composition comprising an attenuated tumor-targeted *Salmonella*, wherein the *Salmonella* comprises a msbB$^-$ mutant. The present specification discloses a mutant msbB$^-$ *Salmonella* strain VNP20009 (page 74, lines 8-9), and the uses of strain VNP20009 together with cytoxan or cisplatin (page 104, line 1 to page 106, line 20).

Claim 123 is drawn to a method of inhibiting the growth of a solid tumor cancer, comprising administering to a subject an effective amount of an anti-cancer compound and an effective amount

of a pharmaceutical composition comprising an attenuated tumor-targeted *Salmonella*, wherein the *Salmonella* comprises a msbB⁻ mutant. The present specification discloses a mutant msbB⁻ *Salmonella* strain VNP20009 (page 74, lines 8-9), and the uses of strain VNP20009 together with an anti-cancer compound (page 104, line 1 to page 106, line 20).

VI. <u>GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL</u>

Whether claims 113, 115-117, and 119-124 were properly rejected under 35 U.S.C. 103(a) as being unpatentable over Low et al. (Nature Biotechnology 1999; 17:155-64) (hereinafter "Low") in view of Schachter et al. (Cancer Biotherapy Radiopharmacology 1998; June: 13:155-64) (hereinafter "Schacter").

VII. <u>ARGUMENT</u>

The Final Office action of December 20, 2007 rejected claims 113, 115-117, and 119-124 over the combined disclosures of Low and Schachter. The Final Office action states the following:

(a) Low teaches a method of treating tumors using an *msbB⁻ Salmonella* mutant which is attenuated and tumor-targeting.

(b) Low teaches that such mutant may be administered to reduce the volume of melanoma solid tumors in a variety of subjects of different species.

(c) Tests conducted by the investigators of the Low reference imply that the aforementioned bacteria can be safe for human use.

(d) Schachter disclosed a routine regimen of chemotherapy comprising the chemotherapeutic agent cisplatin for treating human melanoma, and that the chemotherapeutic regimen had been clinically routine and therefore well-known in the art.

(e) Schachter provides evidence that it is routine to combine chemotherapeutic therapies with newly developed biotherapies, and specifically, in the case of Schachter, a chemotherapy with a biotherapy involving cytokines that regulate the immune system of patients with metastatic melanoma. Schachter therefore supplements Low's deficiencies respecting dual modality of treatment.

(f) Because Schacter also applied the cytokine biotherapy and chemotherapy sequentially, it would have been obvious for a person of ordinary skill in the art to apply the tumor-targeting bacteria of the instant application in a sequential manner with a chemotherapy.

(g) Schacter teaches a need for improvement of conventional chemotherapies with respect to response rate and displays that the response rate of patients is greater when a chemotherapy is used in conjunction with a biotherapy.

Applicants submit that the rejection of the claims is sustained by conclusory statements and an articulated reasoning with a rational underpinning has not been presented to support the legal conclusion as to obviousness.

Claims 113, 115-117, 119-122

The framework for the analysis of obviousness has been settled law. Three factual inquiries must be made to support obviousness:

1) establishing the scope and content of the prior art;
2) establishing the scope of the prior art and the claims at issue; and
3) establishing the level of ordinary skill in the pertinent art.

Last year, the U.S. Supreme Court reiterated the same in KSR International Co. v. Teleflex Inc. and elaborated on the standards for determining obviousness. *See generally* KSR International Co. v. Teleflex, 127 S.Ct. 1727 (2007) (hereinafter "KSR"). The Supreme Court also further iterated that, "[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." KSR at 1741 (quoting In re Kahn, 441 F.3d 977, 988 (CA Fed. 2006)).

Following this decision, the USPTO issued supplemental guidelines for examination. *See* Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR* v. *Teleflex, Inc.*, 72 Fed. Reg. 57526 (October 10, 2007) (hereinafter "Examination Guidelines"). Applicants acknowledge that the USPTO has stated that these

guidelines do not constitute substantive rulemaking and do not have the force and effect of law. Applicants do, however, believe that said guidelines offer evidence that an articulated reasoning with a rational underpinning for obviousness rejection is lacking with respect to the examination of this application.

The USPTO guidelines recite a non-exclusive list of examples of rationales which may be used to articulate a rejection based on obviousness, now at MPEP § 2143. These include:

(A) Combining prior art elements according to known methods to yield predictable results;

(B) Simple substitution of one known element for another to obtain predictable results;

(C) Use of known technique to improve similar devices (methods, or products) in the same way;

(D) Applying a known technique to a known device (method, or product) ready for improvement to yield predictable results;

(E) "Obvious to try" - choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success;

(F) Known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been predictable to one of ordinary skill in the art;

(G) Some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention. *See* Examination Guidelines at 57529.

A review of this list suggest that the Examiner has attempted to employ certain of these arguments but the logic of the arguments is flawed, as demonstrated in the following section of this brief.

1. <u>The Examiner's Stated Rationales Fail to Articulate Grounds for a "Reasonable Expectation of Success" or "Anticipated Success" When Combining the Teachings of Schachter and Low, and are Conclusory</u>

In order to establish a *prima facie* case of obviousness in this matter, it must be shown that as of

Appellants' earliest filing date, the combination of Schachter and Low would have provided a reasonable expectation of success in arriving at the claimed subject matter. MPEP 2142 (*citing, In re* Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). KSR reemphasized this by stating, "When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the **anticipated success**, it is likely the product not of innovation but of ordinary skill and common sense." [Emphasis added.] KSR at 1742. As discussed below, the Examiner's Rationales fail to articulate grounds for a reasonable expectation of success or for anticipated success.

It appears that the Examiner has primarily attempted to frame the combination of Schachter and Low as, "Combining prior art elements according to known methods to yield predictable results," or alternatively, as "'Obvious to try' - choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success." In the Office Action of December 20, 2007, the Examiner makes the following argument against the applicants:

> Again, the instant situation is amenable to the type of analysis set forth in In re Kerkhoven, 205 USPQ 1069 (CCPA 1980), wherein the court held that it is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose in order to produce a third composition that is to be used for the very same purpose since the idea of combining them flows logically from their having been individually taught in the prior art. [December 20, 2007 Office Action, p. 8.]

The Examiner further states:

> Given the teaching of the prior art compositions of cisplatin and attenuated *Salmonella*-all taught to be useful for the treatment of cancer, it would have been *prima facie* obvious to one of ordinary skill in the art to combine these compositions to generate a new composition for the treatment of cancer with a reasonable expectation of success. [Emphasis added] [December 20, 2007 Office Action, p. 8.]

And later cites KSR:

> In response to the argument that there is no specific suggestion or teaching in the

> references to combine prior art, KSR forecloses the argument that a specific teaching, suggestion or motivation is required to support a fining [sic] of obviousness. [...] Here, all the recited elements were known in the art, and hence "THE COMBINATION OF FAMILIAR ELEMENTS ACCORDING TO KNOWN METHODS IF LIKELY TO BE OBVIOUS WHEN IT DOES NO MORE THAN YIELD PREDICTABLE RESULTS." *KSR*, 127 S. CT. AT 1740, 82 USPQ2D AT 1395-96.

However, contained nowhere in the Examiner's rejections is there any rationale that results were "predictable" beyond the conclusory allegation that there would be a reasonable expectation of success when combining an anti-cancer chemotherapy with an anti-cancer biotherapy, generally. *See* December 20, 2007 Office Action, p. 5-6 (stating "*Schachter et al* further supplemented *Low et al* by illustrating it was routine to combine a chemotherapeutic regimen with a newly developed biotherapy in treating solid tumors such as melanoma. [...] [I]t would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the attenuated tumor-targeted mutant *Salmonella* therapy as taught by *Low et al* with a routine chemotherapeutic regimen as taught by *Schacter et al* with a reasonable expectation of success.").

2. <u>A Person of Ordinary Skill in the Art Would Not Have Had a Reasonable Expectation of Success With Respect to the Combination of Low and Schachter As of the Date of Invention</u>

At the time the present application for patent was filed, it was generally believed that there is a problem of *Salmonella* infection of various different strains in those patients with weakened immune systems, such as cancer patient populations subjected to chemotherapeutic regimens. Given that bacterial infection, and bacterial infection with *Salmonella*, was a well-known occurrence among immune-compromised cancer patients undergoing chemotherapeutic therapy, it would have been particularly counterintuitive to combine a live *Salmonella* bacterium shown to infect tumor-tissue with a conventional chemotherapy at the time of invention. Therefore, there could not have been a reasonable expectation of success respecting the combination of a conventional chemotherapy with the disclosed biotherapy of the instant application.

3. The Stated Need in the Field of Endeavor is Overbroad, the Scope and Content of the Prior Art is Overbroad, and the Obviousness Rationale Fails to Evidence Predictability

Under KSR, "[A]ny need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the element in the manner cited." KSR at 1742. This is echoed in rationale (F) which states that obviousness is present where "Known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been predictable to one of ordinary skill in the art." MPEP § 2143.

Here, the Examiner provides an extremely broad problem to be addressed: "The rationale for the design of the combined therapy was to achieve a higher percentage of a complete response (CR, meaning disappearance of all measurable disease) to drug treatment." Final Office Action of December 20, 2007, p. 5. It is again emphasized that the need (as identified in Schachter) is one of "further improvement of the conventional chemotherapy." *Id.* at 6.

Under the Examiner's rationale, nearly any combination of therapies, however novel their use together might be at the time of invention, would be considered obvious if each separately performed a stated function of decreasing measurable disease. Applicants believe that if an obviousness claim were to succeed on such a broad rationale, experimentation in the chemotherapeutic and biotherapeutic arts would be stifled. Moreover, in the explanation of rationale (F), the MPEP states that, the Examiner should show that the scope and content of the prior art, whether in the same field of endeavor as that of the applicant's invention or a different field of endeavor, included a similar or analogous device (method, or product). Applicants would attest that the devices of the prior art are not to be considered "analogous" simply because both are used to treat cancer (and solid tumors particularly). Applicants attest that the biotherapy of Schachter (i.e. a cytokine molecular therapy of interferon-α and GM-CSF), is too different from the biotherapy technology of the instant invention, i.e. a living, replicating bacteria, to form the basis of an obviousness rejection.

No incentives or market forces are identified beyond the generalized goal of improving

chemotherapy and eliminating disease states, and as stated infra, the combination would not have been predictable at the time of invention.

Applicants are aware that under KSR, an analysis by the Examiner of whether there was an apparent reason to combine known elements "need not seek out precise teachings directed to the challenged claim's specific subject matter". KSR at 1741. However, Applicants maintain that the Examiner should show at minimum, more than a general motivation to improve the chemotherapeutic arts, or to combine chemotherapy with any new biotherapy; instead a motivation to combine a chemotherapy with a tumor-targeted *Salmonella* bacteria should be demonstrated.

Claims 123-124

The framework for the analysis of obviousness has been settled law. Three factual inquiries must be made to support obviousness:

1) establishing the scope and content of the prior art;
2) establishing the scope of the prior art and the claims at issue; and
3) establishing the level of ordinary skill in the pertinent art.

Last year, the U.S. Supreme Court reiterated the same in KSR International Co. v. Teleflex Inc. and elaborated on the standards for determining obviousness. *See generally* KSR International Co. v. Teleflex, 127 S.Ct. 1727 (2007) (hereinafter "KSR"). The Supreme Court also further iterated that, "[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." KSR at 1741 (quoting In re Kahn, 441 F.3d 977, 988 (CA Fed. 2006)).

Following this decision, the USPTO issued supplemental guidelines for examination. *See* Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR* v. *Teleflex, Inc.*, 72 Fed. Reg. 57526 (October 10, 2007) (hereinafter "Examination Guidelines"). Applicants acknowledge that the USPTO has stated that these guidelines do not constitute substantive rulemaking and do not have the force and effect of law.

Applicants do, however, believe that said guidelines offer evidence that an articulated reasoning with a rational underpinning for obviousness rejection is lacking with respect to the examination of this application.

The USPTO guidelines recite a non-exclusive list of examples of rationales which may be used to articulate a rejection based on obviousness, now at MPEP § 2143. These include:

(A) Combining prior art elements according to known methods to yield predictable results;

(B) Simple substitution of one known element for another to obtain predictable results;

(C) Use of known technique to improve similar devices (methods, or products) in the same way;

(D) Applying a known technique to a known device (method, or product) ready for improvement to yield predictable results;

(E) "Obvious to try" - choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success;

(F) Known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been predictable to one of ordinary skill in the art;

(G) Some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention. *See* Examination Guidelines at 57529.

A review of this list suggest that the Examiner has attempted to employ certain of these arguments but the logic of the arguments is flawed, as demonstrated in the following section of this brief.

1. <u>The Examiner's Stated Rationales Fail to Articulate Grounds for a "Reasonable Expectation of Success" or "Anticipated Success" When Combining the Teachings of Schachter and Low, and are Conclusory</u>

In order to establish a *prima facie* case of obviousness in this matter, it must be shown that as of Appellants' earliest filing date, the combination of Schachter and Low would have provided a

reasonable expectation of success in arriving at the claimed subject matter. MPEP 2142 (*citing, In re* Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). KSR reemphasized this by stating, "When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the **anticipated success**, it is likely the product not of innovation but of ordinary skill and common sense." [Emphasis added.] KSR at 1742. As discussed below, the Examiner's Rationales fail to articulate grounds for a reasonable expectation of success or for anticipated success.

It appears that the Examiner has primarily attempted to frame the combination of Schachter and Low as, "Combining prior art elements according to known methods to yield predictable results," or alternatively, as "'Obvious to try' - choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success." In the Office Action of December 20, 2007, the Examiner makes the following argument against the applicants:

> Again, the instant situation is amenable to the type of analysis set forth in In re Kerkhoven, 205 USPQ 1069 (CCPA 1980), wherein the court held that it is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose in order to produce a third composition that is to be used for the very same purpose since the idea of combining them flows logically from their having been individually taught in the prior art. [December 20, 2007 Office Action, p. 8.]

The Examiner further states:

> Given the teaching of the prior art compositions of cisplatin and attenuated *Salmonella*-all taught to be useful for the treatment of cancer, it would have been *prima facie* obvious to one of ordinary skill in the art to combine these compositions to generate a new composition for the treatment of cancer with a reasonable expectation of success. [Emphasis added] [December 20, 2007 Office Action, p. 8.]

And later cites KSR:

> In response to the argument that there is no specific suggestion or teaching in the references to combine prior art, KSR forecloses the argument that a specific teaching, suggestion or motivation is required to support a fining [sic] of obviousness. [...]

> Here, all the recited elements were known in the art, and hence "THE COMBINATION OF FAMILIAR ELEMENTS ACCORDING TO KNOWN METHODS IF LIKELY TO BE OBVIOUS WHEN IT DOES NO MORE THAN YIELD PREDICTABLE RESULTS." *KSR*, 127 S. CT. AT 1740, 82 USPQ2D AT 1395-96.

However, contained nowhere in the Examiner's rejections is there any rationale that results were "predictable" beyond the conclusory allegation that there would be a reasonable expectation of success when combining an anti-cancer chemotherapy with an anti-cancer biotherapy, generally. *See* December 20, 2007 Office Action, p. 5-6 (stating "*Schachter et al* further supplemented *Low et al* by illustrating it was routine to combine a chemotherapeutic regimen with a newly developed biotherapy in treating solid tumors such as melanoma. [...] [I]t would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the attenuated tumor-targeted mutant *Salmonella* therapy as taught by *Low et al* with a routine chemotherapeutic regimen as taught by *Schacter et al* with a reasonable expectation of success.").

2. <u>A Person of Ordinary Skill in the Art Would Not Have Had a Reasonable Expectation of Success With Respect to the Combination of Low and Schachter As of the Date of Invention</u>

At the time the present application for patent was filed, it was generally believed that there is a problem of *Salmonella* infection of various different strains in those patients with weakened immune systems, such as cancer patient populations subjected to chemotherapeutic regimens. Given that bacterial infection, and bacterial infection with *Salmonella*, was a well-known occurrence among immune-compromised cancer patients undergoing chemotherapeutic therapy, it would have been particularly counterintuitive to combine a live *Salmonella* bacterium shown to infect tumor-tissue with a conventional chemotherapy at the time of invention. Therefore, there could not have been a reasonable expectation of success respecting the combination of a conventional chemotherapy with the disclosed biotherapy of the instant application.

3. <u>The Stated Need in the Field of Endeavor is Overbroad, the Scope and Content of the Prior Art is Overbroad, and the Obviousness Rationale Fails to Evidence Predictability</u>

Under <u>KSR</u>, "[A]ny need or problem known in the field of endeavor at the time of invention and

addressed by the patent can provide a reason for combining the element in the manner cited." KSR at 1742. This is echoed in rationale (F) which states that obviousness is present where "Known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been predictable to one of ordinary skill in the art." MPEP § 2143.

Here, the Examiner provides an extremely broad problem to be addressed: "The rationale for the design of the combined therapy was to achieve a higher percentage of a complete response (CR, meaning disappearance of all measurable disease) to drug treatment." Final Office Action of December 20, 2007, p. 5. It is again emphasized that the need (as identified in Schachter) is one of "further improvement of the conventional chemotherapy." *Id.* at 6.

Under the Examiner's rationale, nearly any combination of therapies, however novel their use together might be at the time of invention, would be considered obvious if each separately performed a stated function of decreasing measurable disease. Applicants believe that if an obviousness claim were to succeed on such a broad rationale, experimentation in the chemotherapeutic and biotherapeutic arts would be stifled. Moreover, in the explanation of rationale (F), the MPEP states that, the Examiner should show that the scope and content of the prior art, whether in the same field of endeavor as that of the applicant's invention or a different field of endeavor, included a similar or analogous device (method, or product). Applicants would attest that the devices of the prior art are not to be considered "analogous" simply because both are used to treat cancer (and solid tumors particularly). Applicants attest that the biotherapy of Schachter (i.e. a cytokine molecular therapy of interferon-α and GM-CSF), is too different from the biotherapy technology of the instant invention, i.e. a living, replicating bacteria, to form the basis of an obviousness rejection.

No incentives or market forces are identified beyond the generalized goal of improving chemotherapy and eliminating disease states, and as stated infra, the combination would not have been predictable at the time of invention.

Applicants are aware that under KSR, an analysis by the Examiner of whether there was an apparent reason to combine known elements "need not seek out precise teachings directed to the challenged claim's specific subject matter". KSR at 1741. However, Applicants maintain that the Examiner should show at minimum, more than a general motivation to improve the chemotherapeutic arts, or to combine chemotherapy with any new biotherapy; instead a motivation to combine a chemotherapy with a tumor-targeted *Salmonella* bacteria should be demonstrated.

CONCLUSION

In conclusion, it is respectfully suggested that the Honorable Board reverse the appealed rejections as not establishing a *prima facie* case of obviousness. As described at length above, proper application of the methods for determining the motivation to combine the teachings and suggestions of Schachter with those of Low do not rise to the level of *prima facie* obviousness. In large part, the lack of motivation to combine the references is based on the fact that persons of ordinary skill in the art would not have had a reasonable expectation of success in arriving at the appealed claims, as of Appellant's earliest filing date.

Accordingly, reversal of all rejections under § 103(a) is respectfully requested. If any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

Albert Wai Kit Chan

Albert Wai-Kit Chan
Registration No. 36,479
Attorney for Applicants
Law Offices of Albert Wai-Kit Chan, PLLC
World Plaza, Suite 604
141-07 20th Avenue
Whitestone, New York 11357
Tel: (718) 799-1000
Fax: (718) 357-8615
Email:chank@kitchanlaw.com

VIII. CLAIMS APPENDIX

113. (Previously presented) A method of inhibiting the growth of, or reducing the volume of a solid tumor cancer, comprising administering to a subject having a solid tumor cancer an effective amount of cytoxan or cisplatin and an effective amount of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an attenuated tumor-targeted *Salmonella*, wherein the *Salmonella* comprises an msbB$^-$ mutant.

115. (Previously presented) The method of claim 113 wherein, the solid tumor or cancer is either a lung cancer or colon cancer.

116. (Previously presented) The method of claim 113 wherein the subject is a mammal.

117. (Previously presented) The method of claim 113 wherein the subject is a human.

119. (Previously presented) The method of claim 113, comprising administering an effective amount of cisplatin.

120. (Previously presented) The method of claim 113, wherein the administering of the attenuated tumor-targeted *Salmonella* msbB$^-$ and the administering of the cytoxan or cisplatin are not performed concomitantly.

121. (Previously presented) The method of claim 115, wherein the solid tumor cancer is a lung cancer.

122. (Previously presented) The method of claim 115, wherein the solid tumor cancer is a lung cancer.

123. (Previously presented) A method of inhibiting the growth of, or reducing the volume of a solid tumor cancer, comprising administering to a subject having a solid tumor cancer an effective amount of,

(a) a pharmaceutical composition consisting essentially of an anti-cancer compound and one or more pharmaceutically acceptable carriers, and

(b) an effective amount of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an attenuated tumor-targeted *Salmonella*, wherein the *Salmonella* comprises an msbB$^-$ mutant.

124. (Previously presented) The method of claim 123 wherein the anti-cancer compound is cisplatin.

IX. EVIDENCE APPENDIX

Exhibit 1: Chow, et al., In AML cell lines Ara-C combined with purine analogues is able to exert synergistic as well as antagonistic effects on proliferation, apoptosis and disruption of mitochondrial membrane potential. Leuk. Lymphoma 44:165-73 (2003) (Abstract only).

Exhibit 2: Budman et al., Synergistic and antagonistic combinations of drugs in human prostate cancer cell lines *in vitro*. Anticancer Drugs 13:1011-1016 (2002) (Abstract only).

Exhibit 3: Dasmahapatra et al., *In vitro* combination treatment with perifosine and UCN-01 demonstrates synergism against prostate (PC-3) and lung (A549) epithelial adenocarcinoma cell lines. Clinical Cancer Res. 10:5242-5252 (2004).

Exhibit 4: Freifeld et al., Fever in the Neutropenic Cancer Patient, Chap. 46, Clinical Oncology, (3rd ed.) (2004).

<u>Applicant's statement</u>
Exhibits 1-3 were submitted in a response filed March 29, 2007. Exhibit 4 was submitted in a response filed May 24, 2007. The May 24, 2007 response also included copy of the March 29, 2007 response.

In an office action mailed June 28, 2007, the Examiner stated on page 2: "Applicant's submission filed on 5/24/07 has been entered" and "The arguments in 3/29/07 and 5/24/07 response would be addressed ..."

EXHIBIT 1

1: Leuk Lymphoma. 2003 Jan;44(1):165-73. Links

In AML cell lines Ara-C combined with purine analogues is able to exert synergistic as well as antagonistic effects on proliferation, apoptosis and disruption of mitochondrial membrane potential.

Chow KU, Boehrer S, Napieralski S, Nowak D, Knau A, Hoelzer D, Mitrou PS, Weidmann E.

Department of Internal Medicine III, Hematology and Oncology, Johann Wolfgang Goethe-University, University Hospital, Theodor-Stern-Kai 7, 60590 Franfurt/Main, Germany. chow@em.uni-frankfurt.de

The pyrimidine analogue Ara-C and the purine analogues fludarabine and cladribine (2-CdA) are essential compounds in the treatment of acute myeloid leukemia (AML). Inhibition of cell proliferation and induction of apoptosis are the major mechanisms of cytotoxic agents to cause tumor cell death. Therefore, we studied whether Ara-C in combination with the purine analogues exerts synergistic or antagonistic effects on cell proliferation, phosphatidylserine exposure and disruption of mitochondrial membrane potential (MMP) in the AML cell lines HL60 and HEL. Furthermore, effects of the combination of Ara-C with bendamustine, a new bifunctional agent with alkylating activity and a purine nucleus, was investigated. Assessment by combination index analysis showed that Ara-C combined with fludarabine or bendamustine exhibited additive to antagonistic effects on inhibition of cell proliferation, induction of apoptosis as well as on disruption of mitochondrial membrane potential, independent of a simultaneous or consecutive (purine analogues before Ara-C) incubation schedule. In contrast, the combination of Ara-C with 2-CdA exclusively yielded synergistic effects. While inducing IC50 levels of apoptosis neither the antagonistic nor the synergistic drug combinations caused a specific expression pattern of apoptosis-associated proteins such as the pro- or antiapoptotic Bcl-2 family members, executioner caspases, IAPs (inhibitor of apoptosis proteins), proapoptotic Par-4, PARP, or p53. In conclusion, we here demonstrate that the in vitro efficacy of drug combinations containing Ara-C and purine analogues depends on the purine analogue applied, whereas incubation schedules or escalating dosages do not contribute to the synergistic effects.

PMID: 12691159 [PubMed - indexed for MEDLINE]

Related Links

Synergistic effects of chemotherapeutic drugs in lymphoma [Biochem Pharmacol. 2003]

Reversal of cytosine arabinoside (ara-C) resistance [Cancer Chemother Pharmacol. 2001]

In vitro induction of apoptosis of neoplastic cells in low-grade non-h [Haematologica. 2001]

Induction of apoptosis using 2',2' difluorodeoxycytidine (ge [Ann Hematol. 2000]

Intracellular pharmacodynamic studies of the synerg [Cancer Chemother Pharmacol. 1995]

See all Related Articles...

EXHIBIT 2

1: Anticancer Drugs. 2002 Nov;13(10):1011-6.

Synergistic and antagonistic combinations of drugs in human prostate cancer cell lines in vitro.

Budman DR, Calabro A, Kreis W.

Don Monti Division of Oncology, North Shore University Hospital, New York University School of Medicine, Manhasset, NY 11030, USA. budman@nshs.edu

Microtubulin binding agents such as docetaxel have significant preclinical and clinical activity in the treatment of hormone-refractory prostate cancer. We have previously used median-effect analysis to define both synergistic and antagonistic drug combinations which may be of value in management of human disease. These studies extend our findings in defined prostate cancer cell lines. A semi-automated microtiter culture system was used. Docetaxel was combined with 18 other agents, incubated with DU 145, LnCaP or PC 3 prostate cancer cell lines for 72 h and the cells then incubated with MTT to determine cytotoxic effect. Both doublet and triplet combinations were examined. Synergy and antagonism as measured by the combination index were determined for each combination. The non-mutually exclusive criterion was applied. Docetaxel demonstrated cytotoxic additive effects or synergy with -retinoic acid, cyclosporin A and vinorelbine in all three cell lines. Docetaxel combined with either epirubicin or doxorubicin displayed cytotoxic synergistic effects in hormone-refractory DU 145 and PC 3 cell lines. In contrast, drugs which have been combined clinically to treat hormone-refractory prostate cancer, i.e. cisplatin, carboplatin or etoposide, were antagonistic when combined with docetaxel. We conclude that combinations of docetaxel with either -retinoic acid or vinorelbine may offer an enhanced cytotoxic effect in the management of hormone-refractory prostate cancer and need to be evaluated for therapeutic effect. The combination of docetaxel with an anthracycline was also synergistic in the two hormone-refractory cell lines, DU 145 and PC3, thus suggesting a potential role in advanced disease after endocrine failure. Combinations of docetaxel with platinum or etoposide may lead to subadditive effects in treatment.

PMID: 12439335 [PubMed - indexed for MEDLINE]

Related Links

In vitro search for synergy and antagonism: evaluation of dc [Breast Cancer Res Treat. 2002]

In vitro cytotoxic effects of imatinib in combination with anticancer ([Prostate. 2005]

Unique synergism or antagonism of combinations of chemothera| [Br J Urol. 1997]

Zoledronic acid (Zometa) enhances the cytotoxic effect of gemcitabi [Oncology. 2006]

In vitro assessment of cytotoxic agent combinations for horn [Anticancer Drugs. 2005]

See all Related Articles...

EXHIBIT 3

In vitro Combination Treatment with Perifosine and UCN-01 Demonstrates Synergism against Prostate (PC-3) and Lung (A549) Epithelial Adenocarcinoma Cell Lines

Girija P. Dasmahapatra,[1] Parijat Didolkar,[1] Michael C. Alley,[2] Somiranjan Ghosh,[1] Edward A. Sausville,[1] and Krishnendu K. Roy[1]

[1]Clinical Trials Unit and [2]Biological Testing Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, Maryland

ABSTRACT

Purpose: **Antineoplastic agents often achieve antitumor activity at the expense of close to unacceptable toxicity. One potential avenue to improve therapeutic index might combine agents targeting distinct components of the same growth regulatory pathway. This might lead to more complete modulation of the target pathway at concentrations lower than those associated with limiting adventitious toxicities from either agent alone. The protein kinase antagonist UCN-01 is currently used in Phase I/II trials and has recently been demonstrated to inhibit potently PDK1 (S. Sato *et al.,* Oncogene, *21:* 1727–1738, 2002). We have recently documented that the alkylphospholipid perifosine potently also inhibits Akt kinase (PKB) activation by interfering with membrane localization of Akt (S. Kondapaka *et al.,* Mol. Cancer Ther., *2:* 1093–1103, 2003). This leads to the hypothesis that these two agents might act synergistically through distinct mechanisms in the PI3K/Akt proliferation and survival-related signaling pathway.**

Experimental Design: **The synergistic effects of UCN-01 and perifosine, on two cell lines (A-549 and PC-3), were examined using various long-term *in vitro* assays for cell growth, cell cycle distribution, clonogenicity, survival morphology, and apoptosis. Along with Western blotting experiments were performed to determine whether this synergistic combination of two drugs has significant effect on their downstream targets and on biochemical markers of apoptosis.**

Results: **After 72 h, perifosine at concentrations of 1.5 and 10 μM UCN-01 at 40 and 250 nM did not significantly affect the growth of PC-3 and A459 cells, respectively. However, in combination at the same respective individual concentrations (1.5 μM and 40 nM of perifosine and UCN-01, respectively, in PC-3 cells and 10 μM perifosine and 0.25 μM UCN-01 in the somewhat more resistant A549 cells), virtually complete growth inhibition of both the cell lines resulted. Supra-additive inhibition of growth was also demonstrated in independent clonogenic assays. Mechanistic studies in cell culture models suggest enhanced depletion of the S-phase population in cells treated by the combination. This correlated with enhanced inactivation of Akt along with activation of caspases 3 and 9 and poly(ADP-ribose) polymerase cleavage. Evidence of synergy was formally demonstrated and occurred across a wide range of drug concentrations and was largely independent of the order or sequence of drug addition.**

Conclusions: **As the concentrations of UCN-01 and perifosine causing synergistic inhibition of cell growth are clinically achievable without prominent toxicity, these data support the development of clinical studies with this combination.**

INTRODUCTION

Alkylphospholipids have been synthesized with the goal of modifying membrane-related signal transduction events. Hexadecylphosphocholine (miltefosine; Miltex), one of the first of this class to undergo clinical evaluation, had evidence of immunomodulatory and antitumor activity in model systems (1–3). Although low oral bioavailability, gastrointestinal, and hemolytic toxicities limited its clinical development as an anticancer agent, it is approved for use in Europe for topical treatment of cutaneous lymphomas and breast cancer metastases (4) and has activity against visceral leishmaniasis despite low oral absorption (5).

Perifosine [octadecyl-(1,1-dimethyl-piperidino-4-yl) phosphate; D-21266; NSC639966] is an analogue of miltefosine with greater oral bioavailability currently undergoing clinical evaluation (6). Alkylphospholipids are known to alter several aspects of cell membrane synthesis and function, including inhibition of phospholipase C, guanine nucleotide-binding protein, protein kinase C (PKC) activity, and phosphatidylcholine synthesis (7). We recently documented that one consequence of perifosine action is cell cycle arrest with induction of $p21^{WAF1/CIP1}$ in a p53-independent fashion (8). In seeking to define further the pathway leading to $p21^{WAF1/CIP1}$ induction, others and we have recently demonstrated that perifosine causes rapid decrease in the phosphorylation of Akt (PKB), with loss of Akt activity (9, 10) and decreased translocation of Akt to the plasma membrane (10).

UCN-01 (7-hydroxystaurosporine) is a PK antagonist originally defined as a potent selective inhibitor of PKC isoforms α, β, and γ (11–14). It also causes cell cycle arrest in a way that appears to be independent of effects on PKC, with evidence of antitumor activity in model systems (15, 16). It currently is being evaluated in early-phase clinical trials (17). Additional

Received 11/5/03; revised 3/23/04; accepted 4/8/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: G. Dasmahapatra and P. Didolkar contributed equally.

Requests for reprints: Krishnendu K. Roy, NIH, 10 Center Drive, Building 10, Room 6N 113, Bethesda, MD 20892-5680. Phone: (301) 496-4119; Fax: (301) 480-7456; E-mail: kr91w@nih.gov.

cellular targets of UCN-01 include the cell cycle checkpoint kinases chk1 (18, 19) and possibly chk2 (20, 21) with sensitization of treated cells to DNA damage. Recently, Sato *et al.* (22) documented that an additional target of UCN-01 is the phosphatidylinositide-dependent kinase 1 (PDK1). This finding is of interest because PDK1 phosphorylates Akt Ser^{308} and contributes to the activation of Akt activity after growth factor stimulation of phosphatidylinositol-3′ kinase (PI3K; Refs. 23, 24)

Combining antineoplastic agents has proven to result in several effective regimens in cancer therapy. Current cytotoxic regimens emerged from efforts to match agents with nonoverlapping toxicity to the host. In contrast, combining agents, which act at distinct points in a signal transduction pathway, might allow more efficient blockade of that pathway's activation. As perifosine inhibits Akt activation and localization to the cell membrane (10) and UCN-01 inhibits PDK1 (22), we reasoned that combined treatment with UCN-01 and perifosine might offer a way to inhibit more completely signaling through the PI3K/Akt pathway. We document here that indeed perifosine and UCN-01 synergistically inhibit proliferation of PC-3 prostate carcinoma cells, which have mutated the PTEN tumor suppressor gene and therefore have increased activity of the PI3K/Akt pathway, as well as A549 lung carcinoma cells. This is accompanied by enhanced capacity of the combination to cause inhibition of Akt activation and cell cycle arrest compared with the action of the compounds as single agents. We therefore propose that the combination of UCN-01 and perifosine would represent a rational approach to efficient suppression of PI3K/Akt signaling, which could be readily advanced to clinical trial.

MATERIALS AND METHODS

Antibodies and Reagents. Antibodies against total and phosphorylated Akt (Ser^{473} and Thr^{308}), phos-PDK1 (Ser^{241}) and total PDK, and cleaved caspase-3 and caspase-9, caspase-6, and caspase-8, phos-GSK-3α/β [$Ser^{21/9}$, phos-Chk1 (Ser^{317}), phos-Chk2 (Thr^{68})] are purchased from Cell Signaling Technologies (Beverly, MA). Two other antibodies namely Total Chk1 and Chk2 are purchased from Upstate Biotechnology, (Lake Placid, NY). All cell culture medium and reagents were from Life Technologies, Inc. (Rockville, MD). Perifosine was obtained from Asta Medica and UCN-01 from Kyowa Hakko Kogyo, through the Developmental Therapeutics Program, National Cancer Institute. Unless otherwise noted all other chemicals are from Calbiochem.

Tissue Culture. Cell lines were grown in their respective culture medium as recommended by the American Type Culture Collection. A549 non-small cell lung carcinoma cells are grown in DMEM, and human prostate adenocarcinoma PC-3 cells were maintained in RPMI at 37°C in an atmosphere containing 5% CO_2. In both cases, the media are supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin medium, and 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY).

***In Vitro* Cytotoxicity Assay.** Tumor cells were cultured in 100-mm Petri dishes with initial cell number so that 40–50% confluency would be achieved after 24 h. These exponentially growing cells were exposed to different concentrations of drug for 24 h. The adherent cells were harvested with trypsin, washed with PBS, and collected by centrifugation at 1500 rpm for 5 min. Trypan blue (0.5%), excluding cells, was enumerated by hemocytometer. CalcuSyn software was obtained from BIOSOFT, Inc. (Cambridge, MA). Three sets of experiments for each drug combinations were carried out, one with the combination of perifosine and UCN-01 and one set with each drug alone. The proportions of dead cells in treated plates were entered into the software, and the degree of synergy assessed by the combination index (CI) value was computed automatically to indicate the degree of synergy or antagonism according to the algorithms described by Chou and Talaly (25).

Soft Agar Colony Formation Assays. Bilayer soft agar colony formation assays of A-549 and PC-3 cell lines were performed using RPMI 1640 containing 10% fetal bovine serum and 10,000 cells/2 ml culture on day 0, as previously described by Alley *et al.* (26). For drug sensitivity assays, 0.1 ml of culture medium containing drug (n = 3/each of 8 drug concentrations spanning the effective drug concentration range) and/or drug vehicle (n = 3) were applied to cultures on day 1. Cultures were then incubated until day 7, stained with methylthiazoletetrazolium, stabilized, and clarified with protamine sulfate buffer and then analyzed by computerized image analysis. Percentage of vehicle control (T/C) values were calculated for each drug concentration as well as the IC_{50} (mean + SD).

Cell Cycle Analysis. Cells were cultured in a 100-mm Petri disk and allowed to grow to 75–80% confluency. Then cells are treated with drugs of interest for 24 h and compared with control samples not exposed to drug. After drug exposure, cells were harvested by trypsin, followed by centrifugation at 1000–1500 rpm in a 15-ml tube. Harvested cells were washed twice with 1× PBS and then resuspended and then fixed by adding 4 ml of cold 100% ethanol added in pulses of 1 ml each while vortexing. Samples were stored −20°C for a minimum of 24 h and up to 1 month before analysis. When samples were to be analyzed, these are centrifuged, the ethanol removed, and cell pellets washed twice with 1× PBS and resuspended in 0.5–1 ml of 50 μg/ml propidium iodide (PI) solution in PBS plus 2 μl/1 ml RNase, and incubated for 1 h at 37°C. Cell cycle distribution was analyzed by flow cytometry and analyzed using FACSCaliber (Becton Dickinson Immunocytometry Systems, San Jose, CA), using MODFIT software (Verity Software, Topsham, ME).

Bromodeoxyuridine (BrdUrd) Incorporation. A549 cells were cultured, drug treated, and collected as mentioned above. Before collection, cells were incubated with 10 μM BrdUrd (BrdUrd kit; Sparta Labs, Biocarta San Diego, CA) for 2 h.

Following the manufacturer's instructions, cells were harvested and washed with PBS. Cells were then photolyzed with UVB light for 5 min. Seventy percent ethanol was then used to fix the cells for 1 h. Cells were washed with PBS and incubated at room temperature for 5 min. Subsequently, cells were resuspended in PBS, distilled water, and fluorescence-activated cell sorting buffer (provided with kit). To each sample, 10 μl of anti-BrdUrd FITC and 20 μl of 7-amino-actinomycin D DNA staining reagent were added. Lastly, samples were incubated at room temperature and in the dark for 1 h and then analyzed with flow cytometry.

Apoptosis Assays. Cells were stained using an Apo-DETECT Annexin V-FITC kit (Zymed Laboratories, Inc.) fol-

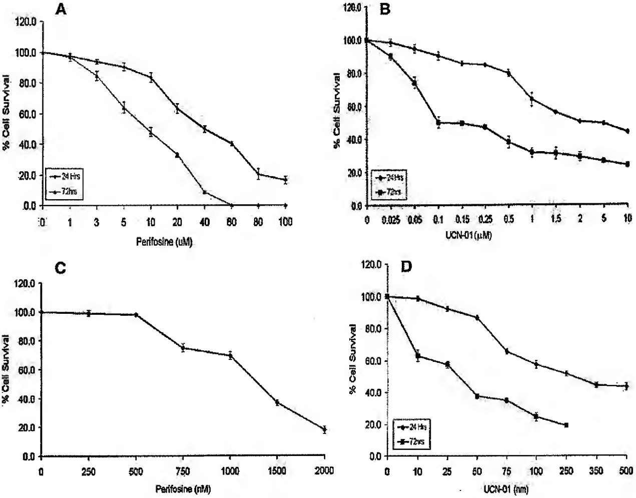


Fig. 1 Effect of perifosine and UCN-01 on growth of A549 and PC-3 cells. A549 cells were plated as described in "Materials and Methods" for the period indicated on the graph. Perifosine (*A*) or UCN-01 (*B*) was added in increasing concentrations and compared with control plates. After specified period of drug exposure (either 24 or 72 h), cells were harvested by trypsinization and centrifuged at 1500 rpm. Cell pellets were resuspended in 1 ml of PBS. A small aliquot is stained with Trypan blue and cells counted by hemocytometer to assess viable cell numbers. The fractions of cells surviving in drug-treated plates are plotted against drug concentration. Similarly, PC-3 cells were treated with perifosine (*C*) or UCN-01 (*D*). In Fig. 1*C* represents, the effect on perifosine to PC-3 cells for 72 h treatment. The value for 24 h experiment has been previously reported (10) and hence not presented. Each statistical *error bar* represents six independent experiments.

lowing the manufacturer's instructions. Briefly, cells were cultured in a 90-mm tissue culture plates (Falcon BD) for the confluency to arrive ~35–40% and then treated with UCN-01 (250 nm), perfosine (10 μm), or a combination of the two for 48 h. A549 cells were released with Trypsin-EDTA 0.25% and were collected and washed with PBS. Following the manufacturer's instruction, cells were stained with Annexin V-FITC and PI. Samples were then processed by flow cytometry. Untreated A549 cells were used as a control. Data acquisition and analysis were done on a BD (Becton Dickinson) FACSCaliber using CellQuest software (BD Biosciences). Annexin V is a calcium dependent phospholipid-binding protein with a high affinity for phosphatidylserine. Phosphatidylserine is normally present in the inner lipid bilayer but becomes exposed on the cell surface within the first few hours of the onset of apoptosis (27). PI is a red DNA-binding dye that can only enter cells whose membranes are disrupted such as in cells undergoing necrosis. Apoptotic cells are stained positive with Annexin V-FITC but are resistant to PI staining.

To assess nuclear morphology, A549 cells were grown on a single well-chambered slide (Labtek) and incubated with UCN-01 (250 nm) or perifosine (10 μm) or a combination of the two for 48 h. Protocol was followed as stated in (28). Combination treatment included both drugs given at the same time, one given for 24 h and then the second added and *vice versa*. Control (nondrug-treated) and drug-treated cells were fixed in 100% cold methanol for 20 min, washed with ice-cold PBS, and then stained for 15 min with 3 μm of Hoechst 33342 dye (Molecular Probes, Eugene, OR). Fluorescent nuclei were visualized in fluorescence microscope (Olympus). Under these conditions, nuclei from living, apoptotic, and necrotic cells could be clearly distinguished. Cells with condensed and deformed nuclei that

Table 1 Analysis of combined treatment of perifosine and UCN-01 in A549 cells: combination index

Perifosine (μM)	UCN-01 (μM)	Combination index		
		Simultaneous	Sequential-I	Sequential-II
6	0.15	0.62 ± 0.017	0.60 ± 0.035	0.32 ± 0.045
8	0.20	0.55 ± 0.02	0.62 ± 0.021	0.31 ± 0.025
10	0.25	0.51 ± 0.013	0.58 ± 0.037	0.34 ± 0.023

NOTE. A constant drug concentration ratio was added to A549 cells (sequential-I–UCN-01 added for 48 h after 24 h of exposure to perifosine; sequential-II-perifosine added for 48 h after 24 h of exposure to UCN-01). The fraction of surviving cells in drug-treated plates is calculated compared with controls. CalcuSyn software is used as described in "Materials and Methods" and the combination index of the different combinations is computed.

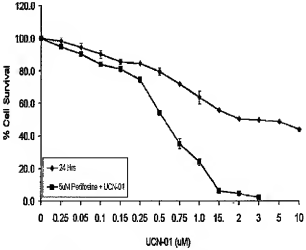


Fig. 2 Sequential treatment of perifosine and UCN-01 on growth of A549 cells for 24 h. Log-phase growing cells were grown in plates as described in "Materials and Methods." Perifosine (5 μM) and an increasing concentration of UCN-01 were added at 50% cell confluency. After 24 h of drug exposure, cells were trypsinized, and viable cells were counted.

showed patches of compact chromatin were considered apoptotic.

Cell Lysis and Immunoblot Analysis. After trypsinization and washing twice with PBS, cells were centrifuged at 1500 rpm and lysed with 50 mM HEPES (pH 7.4), 20 mM EDTA, 0.5 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 1 mM sodium fluoride, 10% glycerol, 0.5% NP40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride. Twenty to 40 μg of total protein were resolved by 4–20% Tris-glycine SDS-PAGE (Novex) gel. The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corp.) in a Hoofer transblotter using 25 mM Tris, 192 mM glycine, and 20% methanol. After the transfer is completed (2–3 h), the blots were blocked for an hour in a blocking buffer containing 5% (w/v) blotto (Santa Cruz Biotechnology, Santa Cruz, CA) in TTBS [10 mM Tris-HCl, 140 mM NaCl (pH 7.4), and 1% (v/v) Tween 20)]. The membranes were washed three times extensively in TTBS. The blots were then placed in their respective primary antibodies at optimal concentrations for 1 h. After three washes with TTBS, the horseradish peroxidase-conjugated specific secondary antibodies were added and additionally incubated for 1 h in presence of 5% (w/v) blotto in TTBS. The membranes were washed extensively in TTBS, and detection was performed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS

Growth Inhibition by Perifosine and UCN-01. The effect of perifosine and UCN-01 as single agents on the growth of the A549 and PC-3 cells was first assessed after 24 and 72 h. Perifosine inhibits A549 cell growth with 50% growth inhibition (IC_{50}) of 40 and 10 μM (Fig. 1*A*) for 24 and 72 h exposures, respectively. Treatment with UCN-01 causes growth inhibition with an IC_{50} of 2 and 0.25 μM (Fig. 1*B*) for the same intervals. In PC-3 cells, the IC_{50} for growth of perifosine is 1.2 μM after 72 h (Fig. 1*C*), whereas after 24 h the IC_{50} is 5 μM [data not shown (10)]. UCN-01 has an IC_{50} for growth of 100 and 40 nM for PC-3 cells in 24 and 72 h, respectively (Fig. 1*D*).

Combination of Perifosine and UCN-01 on the Growth of A549 Cells. To evaluate potential synergy, we added combinations of perifosine and UCN-01 at constant ratios with respect to their respective IC_{50} concentrations. Both drugs were added simultaneously and in separate experiments with one drug preceding the other by 6–8 h. We evaluated synergy using CalcuSyn software to evaluate the combination index originally described by Chou and Talalay (29, 30). Combination index < 1 is evidence for synergy, whereas combination index > 1 is evidence of antagonism, and combination index $= 1$ indicates simple additivity of drug effect. Both simultaneous as well as

Table 2 Analysis of combined treatment of perifosine and UCN-01 in A549 and PC-3 cells: combination index

	Perifosine (μM)	UCN-01 (μM)	Combination index
A	1.0	0.05	0.55 ± 0.024
	2.0	0.05	0.58 ± 0.025
	5.0	0.05	0.40 ± 0.038
	8.0	0.05	0.41 ± 0.031
	10.0	0.05	0.42 ± 0.048
B	2.0	0.05	0.57 ± 0.024
	2.0	0.1	0.52 ± 0.045
	2.0	0.15	0.45 ± 0.021
	2.0	0.20	0.49 ± 0.029
	2.0	0.25	0.50 ± 0.037
C	1.2	0.04	0.51 ± 0.032
	0.9	0.03	0.52 ± 0.035
	0.6	0.02	0.50 ± 0.025
	0.3	0.01	0.42 ± 0.028

NOTE. *A*, a nonconstant drug concentration ratio was added in sequential treatment schedule (perifosine added for 48 h followed by UCN-01 after 24 h in A549 cells); *B*, a nonconstant drug ratio and sequential treatment schedule (UCN-01 added for 48 h followed by perifosine after 24 h in A549 cells); *C*, constant drug ratio and sequential treatment schedule (UCN-01 is added after 24 h of exposure to perifosine in PC-3 cells). The fraction of cell survival in drug-treated plates is calculated with respect to control, and CalcuSyn software was run with relevant data as per in "Materials and Methods," and the combination index of the different combinations is computed.

sequential treatment with perifosine and UCN-01 demonstrates clear evidence of synergy because combination index was <1 in all cases and very often between 0.3 and 0.7 (Table 1). However, the sequential addition of UCN-01 followed by perifosine shows a trend toward improved synergy compared with the reverse sequence of addition or simultaneous exposure to the drugs.

We have also evaluated the combination indexes for combined treatment with UCN-01 and perifosine with a nonconstant drug ratio. In this design, the concentration of one drug is kept constant at its IC_{10} concentration, and the concentration of other drug is then added in successive increments (*i.e.,* in a nonconstant drug ratio). Table 2, *A* and *B*, shows that the combination index, when drugs are added in sequence (perifosine followed by UCN-01 or in reverse sequence), also suggests a synergistic effect of similar magnitude to that seen in the experiments with a constant drug ratio. An example of one result, with perifosine at its IC_{10} (5.0 μM), combined with varying concentrations of UCN-01 (Fig. 2), demonstrates a 3.5-fold decrease of IC_{50} for UCN-01 when added in presence of 5.0 μM perifosine. A similar series of experiments, keeping UCN-01 concentration constant at IC_{10} value (40 nM) with variable concentrations of perifosine has been carried out (data not shown) and comparable decreases in the IC_{50} concentration have been observed.

As perifosine and UCN-01 consistently demonstrated evidence of synergy in the A549 lung carcinoma cells [wild-type p53 and intact *PTEN* gene (31)], we extended a similar set of analyses to PC-3 cells which have mutated p53 (32, 33) and deletion of the PTEN gene product (34) and therefore constitutive activation of the PI3K/Akt pathway. Table 2*C* indicates that similar to A549 cells, PC-3 cells exhibit considerable synergistic effect to both simultaneous, as well as sequential treatment of perifosine and UCN-01, because in all such cases combination index < 1 is obtained and very often lies between 0.4 and 0.5.

To assess by an independent assay the potential value of combined UCN-01 and perifosine treatment, clonogenic unit assays were undertaken. Fig. 3 demonstrates that in both A549 cells (p53 and PTEN both wild type) and PC-3 cells (p53 and PTEN mutant), perifosine at clinically achievable concentrations of 1–10 μM (A549 cells) or 30–300 nM (PC-3 cells) in the presence of 3–30 nM (A549 cells) or 15–45 nM (PC-3 cells) UCN-01, respectively, shows clear evidence of supra-additive diminution of clonogenic potential.

Effect of Combined Perifosine and UCN-01 on Cell Cycle Progression. To begin to understand the basis for synergistic inhibition of cell growth on the part of UCN-01 and perifosine, we studied the effect of the two agents alone (10 μM perifosine and 250 nM UCN-01) or in combination on cell cycle progression. In A549 cells (Fig. 4*A*) in untreated controls, cells are present in G_1 (74%), G_2 (9.4%), and S (16.6%) phases. Concordant with the growth inhibition data, A549 cells are minimally perturbed by either 10 μM perifosine or 250 nM UCN-01, whereas the combination causes a consistent increase in G_2-M (to 18.5%) along with virtual loss of the S-phase (1.3%) fraction. Similarly, cell cycle analysis of the PC-3 cells (Fig. 4*B*) indicates that individual treatment of either perifosine or UCN-01 (at 1.2 and 0.04 μM, respectively) has a marginal effect, whereas combined treatment of perifosine and UCN-01 leads also to virtual loss of the S-phase population and retention of G_1 and G_2 populations for the duration of this experiment. Independent analyses where A549 were plated at lower initial density so that the S-phase fraction of cell cultures was 27.5% at the time of initial exposure to drugs revealed little change after exposure to perifosine (10 μM) for 48 h, partial S-phase suppression (to S-phase fraction of 10%) after exposure to UCN-01 (250 nM), but marked suppression of S-phase fraction (1 to 4%) after combined exposure to 250 nM UCN-01 and 10 μM perifosine (data not shown).

To demonstrate further the capacity of perifosine and UCN-01 to suppress S-phase progression, assessment of BrdUrd incorporation was undertaken. Fig. 5 demonstrates that while 10 μM and 250 nM perifosine and UCN-01, respectively, have little effect as single agents to suppress BrdUrd incorporation, both

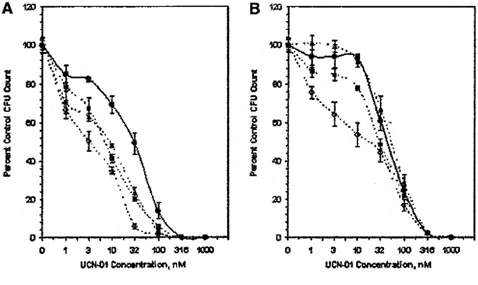


Fig. 3 Combined effect of perifosine and UCN-01 on growth of A549 (*A*) and PC-3 (*B*) cells and soft agar colony forming assay. *A*, concentration, effect profiles of A549 cells exposed to UCN-01 alone (●) and in combination with perifosine [100 nM (△), 316 nM (□), and 1000 nM (◇)]. *B*, concentration, effect profiles of PC-3 cells exposed to UCN-01 alone (●), and in combination with perfosine [31.6 nM (△), 100 nM (□), and 316 nM (◇)]. Data represent the mean ± SD (*n* = 6 for vehicle control-treated cultures and *n* = 3 for cultures treated with each drug concentration).

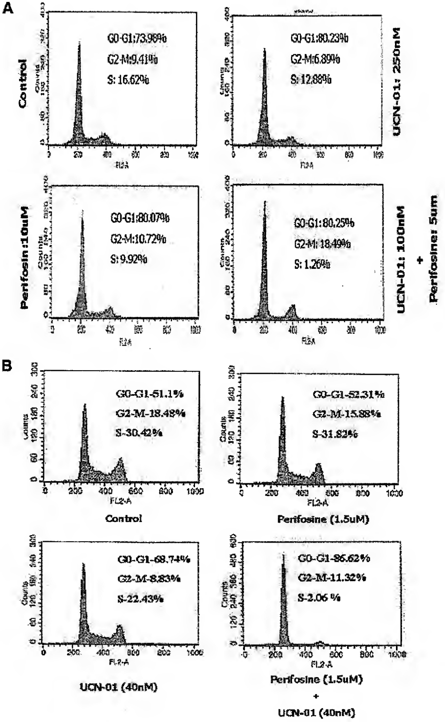


Fig. 4 Cell cycle analysis of combined treatment of perifosine and UCN-01 on A549 (*A*) and PC-3 (*B*) cells. UCN-01 or perifosine was added to A549 and PC-3 cells at ~75–80% confluency and exposed to the drugs for 24 h; cells were then harvested, washed with 1× PBS, and fixed in 100% cold ethanol as explained in "Materials and Methods." Flow cytometry was done to define the cell cycle distribution in comparison to untreated controls.

agents together or in either sequence of addition markedly suppress BrdUrd incorporation.

No significant difference in the type or pattern of cell cycle arrest is observed when combined treatment occurs with simultaneous or sequential drug addition (data not shown). On the basis of these data, we chose concentrations of perifosine (10 μM) and UCN-01 (250 nM) for use in A549 cells to additionally characterize the biochemical correlates of the combined actions of these anticancer agents.

Effect on Akt on Combination Treatment of Perifosine and UCN-01. Fig. 6*A* indicates that exposure of A549 cells individually to perifosine (10 μM) or UCN-01 (250 nM) has no

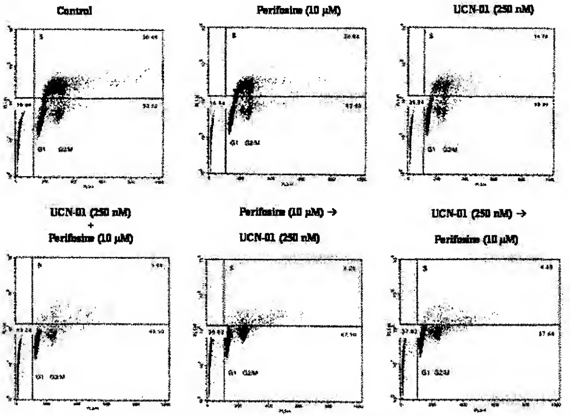


Fig. 5 Combination of UCN-01 and perifosine halts DNA synthesis in A549 cells. Cells were treated with UCN-01 (250 nM), perifosine (10 μM) individually, or a combination of the two with the former given first and the latter given 24 h later, *vice versa* (sequential), or at the same time (simultaneous) and continued for total up to 48 h. Cells incorporated bromodeoxyuridine for 2 h before being harvested. The top right quadrant indicates cells undergoing DNA synthesis (S phase), and the lower left quadrant indicates apoptotic cells by their sub-G_0-G_1 levels of DNA (stained with 7-amino-actinomycin D). The bottom right quadrant indicates cells in either the G_1 or G_2-M phases. The quantified S-phase percentage within the population is indicated.

effect at all on the phosphorylation state of Akt at either Ser^{473} or Thr^{308}. On the other hand, complete dephosphorylation of Akt is observed at both sites when the cells are exposed to the combination of 10 μM perifosine and 250 nM UCN-01 for 24 h when both the drugs are added simultaneously. Similar results are obtained with sequential drug treatment where UCN-01 is added for 18 h followed by perifosine for 6 h of treatment. The level of total Akt remains equivalent to control cells, which suggests that perifosine and UCN-01 cause reduced phosphorylation of Akt without evidence for an effect on the turnover of total Akt.

If Akt dephosphorylation is functionally significant, the phosphorylation or activity of downstream Akt targets such as glycogen synthase kinase (GSK-3) should be diminished. Indeed, we observe considerably less phosphorylation of GSK-3 α/β when cells are treated with the combination of perifosine and UCN-01 in comparison to either treatment alone (Fig. 6*B*). Of interest, Fig. 6*C* shows that P-Chk1 (Ser^{345}), which occurs in relation to replication fork movement (35), shows no diminution of phosphorylation as result of combined UCN-01 and perifosine exposure, in contrast to Akt and GSK-3 isoforms, arguing against a nonspecific effect on phospho-protein status in general.

Combined Perifosine and UCN-01: Effect on Cell Death Pathways. As the Akt pathway is prominently known to affect susceptibility to apoptosis, we assessed whether combined exposure to perifosine and UCN-01 resulted in activation of cell death mechanisms. Fig. 7*B* demonstrates that A549 cells exposed to 10 μM perifosine or 250 nM UCN-01 show minimal changes in nuclear chromatin condensation yet combined exposure or sequential addition of the two agents together causes abundant alteration of nuclear morphology concordant with initiation of apoptosis. Fig. 7*A* quantifies the increase in cellular fraction labeling with Annexin V, which increases from 2.5 and 3.0% in the presence of perifosine and UCN-01, respectively, to 34% with combined exposure. Fig. 7*C* indicates that either perifosine (10 μM) or UCN-01 (250 nM) alone each has no effect on cleaved caspase-3 and caspase-9 production, whereas the combination of both either simultaneously or sequentially at these concentrations causes pronounced cleavage of caspase-3 and caspase-9 after 48 h, suggesting that enhanced cell death by the combination occurs and is mediated through activation of caspase-3 and caspase-9 pathway. In addition, Fig. 7*C* indicated that no activation of caspase-6 and caspase-8, which suggests that cell death through combination drug treatment occurs predominantly through caspase-3- and caspase-9-related pathways.

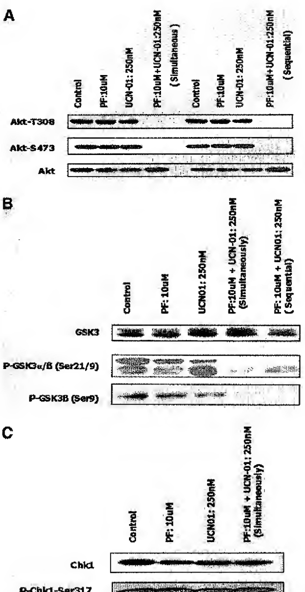


Fig. 6 Combined effect of perifosine and UCN-01 on Akt, glycogen synthase kinase 3 (GSK-3), and Chk1 phosphorylation in A549 cells. Exponentially growing A549 cells in a 100-mm Petri dish were treated with perifosine (10 μM) and UCN-01 (250 nM) for 48 h as indicated in the figure. Drugs are added either the same time (simultaneous) or perifosine followed by 24 h of exposure of UCN-01 (sequential). Cells were lysed, and equal amounts of protein separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with total and phospho-specific Akt (*A*), total and phospho-specific GSK-3 (*B*), and as well as total and phospho-specific Chk1 (*C*) antibodies as described in "Materials and Methods."

As expected, combined exposure also augments poly(ADP-ribose) polymerase cleavage and release of Apaf1, indicating mitochondrial damage, diminution of bcl2 and $bclX_L$, and marked increase in bax expression. Fig. 7*D* indicates that no significant difference on the expression of cell cycle inhibitor proteins such as p27 and p57 Kip1 proteins but, in relation to p21Cip1/Kip1, a modest change is observed when combined treatment occurs with simultaneous or sequential drug addition.

DISCUSSION

In this study, we have shown in cell cultures *in vitro* as well as soft agar colony formation assays that the combination of UCN-01 and perifosine displays substantial synergy in cell growth inhibition of both the p53 wild-type, PTEN wild-type A549 lung carcinoma, and the p53 mutant, PTEN-negative PC-3 prostate carcinoma cell lines. This occurs over a wide range of drug concentration ratios and appears to be relatively independent of the order or sequence of drug addition, although there is a tendency for greater combination effect when UCN-01 preceded perifosine in certain cases. The synergy is manifested in decreased S-phase fraction of treated cultures, loss of Akt activation, and induction of indicators of apoptosis. We therefore propose that additional exploration of this combination in clinical trials is warranted.

Previous studies had shown that PDK1 is a target very sensitive to UCN-01, with subsequent loss of Akt activation and activity (22). Likewise, our recent studies have identified perifosine as a potent inhibitor of Akt activation without a discernible effect on PDK1 intrinsic activity but with a pronounced effect on the recruitment of Akt to the cell membrane (10). Thus, UCN-01 and perifosine could each affect different steps in the pathway, leading to full activation of Akt. Our studies here demonstrate that at very low concentrations of either drug, essentially at concentrations where as single agents they inhibit growth by no more than 10–20%, there is marked interaction in affecting biochemical correlates of Akt activation. These include Akt Thr^{308} and Ser^{473} phosphorylations and Akt activity as measured by phosphorylation of GSK. These combined effects of perifosine and UCN-01 plausibly could contribute to pronounced cell cycle arrest and evidence for induction of apoptosis.

In the experiments studied here, the range of effective concentrations in combinations demonstrating pronounced biochemical effect was 40 nM and 1.5 μM UCN-01 and perifosine, respectively, in PC-3 cells and up to 0.25 and 10 μM, respectively, in the somewhat more resistant A549 cells. Early clinical studies have demonstrated that free UCN-01 not bound to α_1-acid glycoprotein ranged between 50 and 400 nM, with total concentrations of as high as 24 μM at well-tolerated dose levels (36, 37). Likewise, perifosine achieves concentrations of 10–18 μM at well-tolerated doses (38). Thus, the concentrations studied here are clearly within clinically achievable range. Moreover, the relative schedule independence conveys confidence that the relatively prolonged elimination half-lives observed in humans for both agents would actually mimic the conditions used in cell culture here. The side effect profiles of the two drugs as single agents are also distinct. At the respective recommended Phase II doses, prominent effects for UCN-01 include headache, low-grade nausea, and a tendency to hyperglycemia (37). Likewise, for perifosine, gastrointestinal toxicities controlled by antiemetics appear to predominate (38). No other serious end-organ toxicities have emerged.

Although in general it is desirable where possible to obtain evidence of synergistic activity in animal models to extend such

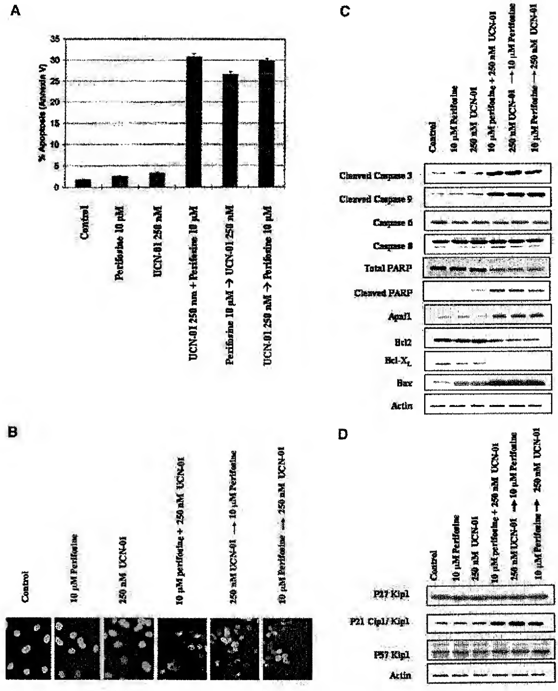


Fig. 7 Combined treatment of perifosine and UCN-01 on the effect of apoptosis on A549 cells. Apoptosis measurement through Annexin V staining (*A*), nuclear morphological evidence (*B*), effect on biochemical markers of apoptosis (*C*), effect on the expression of cell-cycle inhibitor proteins (*D*). *A*, cells were treated with UCN-01 (250 nM), perifosine (10 μM), or a combination (sequential) of the two with the former given first and the latter given 24 h later, *vice versa*, or at the same time (simultaneous) for total of 48 h. The number of apoptotic cells was quantified by FACSCaliber flow cytometry using the CellQuest software after the cells were stained with Annexin V and PI. The chart diagram represents the Annexin V-positive and propidium iodide-negative cells, thus accounting for the percentage of apoptotic cells within the population. *B*, nuclear morphological evidence of apoptosis was demonstrated by using Hoechst 33342 staining after treatment with 10 μM perifosine, 250 nM UCN-01, or a combination of 10 μM perifosine and 250 nM UCN-01 (simultaneous) for 48 h. Other two sequential situations were 10 μM perifosine for 24 h first, followed by 250 nM UCN-01 for another 24 h or *vice versa* order, *i.e.*, 250 nM UCN-01 first for 24 h followed by 10 μM perifosine for another additional 24 h as described under "Materials and Methods." Prominent condensed and deformed nuclei of apoptotic cells are evident on either simultaneous and or sequential treatment of both the compounds. *C* and *D*, exponentially growing A549 cells in a 100-mm Petri dish were treated with perifosine (10 μM) and UCN-01 (250 nM) for 24 h as indicated in the figure. After the completion of treatment, cells were lysed, equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed with respective antibodies as indicated in the figure, and as described in "Materials and Methods."

in vitro results, that goal is problematic in the case of UCN-01 and perifosine because the murine pharmacology of UCN-01 differs considerably from the human because of species-dependent binding to plasma proteins (36, 39). Thus, animal model data of this type would be of uncertain value in reliably modeling the anticipated experience in humans.

There is emerging evidence that several components of the PI3K/Akt/PTEN pathway are involved in oncogenesis (40, 41). Increased activity of the pathway either through amplification or overexpression of PI3K and Akt, activation of growth factor receptor activity activating PI3K, or loss of PTEN activity have been described in many malignancies (42–45). So, the PI3K/Akt/PTEN pathway is an attractive target for drug development as agents directed at the pathway might inhibit cell proliferation and reverse antiapoptosis pathways conveying resistance to cytotoxic therapy in cancer cells. In this regard, UCN-01 shows some promise in inhibiting PDK1 that causes Akt activation (22). Perifosine also causes Akt inactivation most likely by inhibiting its translocation to the plasma membrane (10) where Akt generally is activated by its PDK1 and PDK2 or other upstream kinases. It has been difficult to define clinically useful drugs to interdict this pathway. Wortmannin and derivatives have been extensively considered as inhibitors of PI3K itself. However, toxicity and specificity concerns have precluded extensive development. Although specific inhibitors of PI3K, PDK1, and Akt have not yet reached the clinic, the rapamycin derivatives CCI-779 and RAD001, which inhibit the additional downstream of Akt, mammalian target of rapamycin, are undergoing clinical evaluation but might be viewed as relatively limited in their influence to the more downstream targets of pathway action. Thus, combined UCN-01 and perifosine may be considered a novel approach to down-modulating PI3K/Akt pathway activation in a way that may obviate concerns with the other currently available strategies. In the event that this combination does receive a clinical test, the end points used here (Akt phosphorylation epitopes, GSK activation) might be of value in assessing PI3K/Akt/PTEN pathway activation in surrogate or tumor cells.

Recently, considerable attention has focused on the use of signal transduction modulators together to enhance the lethal effects of cytotoxic agents, building on the groundbreaking observations of enhancement of Herceptin action when in combination with cytotoxics (46–48). In this instance, described here, we provide evidence for the potential value of combining two antisignaling agents. Other combinations of signaling agents have also been recently defined where also the opportunity for enhanced benefit can be discerned. For example, UCN-01 itself has been reported recently to enhance the action of 17-allylamino, 17-demethoxygeldanamycin (49), and of the pharmacological mitogen-activated protein kinase kinase inhibitors (*e.g.*, PD98059 or U0126) in human leukemia (50). Flavopiridol interacts synergistically with imatinib in the inhibition of resistant chronic myelogenous leukemia cell growth (51). All of these efforts are seeking to define a new approach to combination therapy for cancer where the basis for clinical enthusiasm relates not only to acceptable toxicity characteristics of the single agents but where the components of a combination complement each other by modulating distinct steps in a single pathway or in affecting distinct processes important in the development of the neoplasia, *e.g.*, antiproliferative agents combined with antiangiogenic drugs.

In conclusion, taken together, our results demonstrate that the combined effect of perifosine and UCN-01 clearly results in more greatly enhanced antiproliferative action than is seen with either acting singly and that this occurs coincident with enhanced capacity of the combination to down-regulate Akt signaling. As described here, perifosine and UCN-01 share effects on Akt-mediated signaling that point to their combination as biologically plausible by acting at least partly through effects on different aspects of the same proliferation and survival-related signaling pathway and whose human pharmacological features support the feasibility of a strategy amenable to a relatively straightforward clinical test.

REFERENCES

1. Hilgard P, Kampherm E, Nolan L, Pohl J, Reissmann T. Investigation into the immunological effects of miltefosine, a new anticancer agent under development. J Cancer Res Clin Oncol 1991;117:403–8.

2. Rybczynska M, Spitaler M, Knebel NG, Boeck G, Grunicke H, Hofmann J. Effects of miltefosine on various biochemical parameters in a panel of tumor cell lines with different sensitivities. Biochem Pharmacol 2001;62:765–72.

3. Ruiter GA, Zerp SF, Bartelink H, van Blitterswijk WJ, Verheij M. Alkyl-lysophospholipids activate the SAPK/JNK pathway and enhance radiation-induced apoptosis. Cancer Res 1999;59:2457–63.

4. Clive S, Gardiner J, Leonard RC. Miltefosine as a topical treatment for cutaneous metastases in breast carcinoma. Cancer Chemother Pharmacol 1999;44(Suppl):S29–30.

5. Jha TK, Sundar S, Thakur CP, et al. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N Engl J Med 1999;341:1795–800.

6. Crul M, Rosing H, de Klerk GJ, et al. Phase I and pharmacological study of daily oral administration of perifosine (D-21266) in patients with advanced solid tumours. Eur J Cancer 2002;38:1615–21.

7. Maly K, Uberall F, Schubert C, et al. Interference of new alkylphospholipid analogues with mitogenic signal transduction. Anticancer Drug Des 1995;10:411–25.

8. Patel V, Lahusen T, Sy T, Sausville EA, Gutkind JS, Senderowicz AM. Perifosine, a novel alkylphospholipid, induces p21(WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest. Cancer Res 2002;62:1401–9.

9. Ruiter GA, Zerp SF, Bartelink H, Van Blitterswijk WJ, Verheij M. Anti-cancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase-Akt/PKB survival pathway. Anticancer Drugs 2003;14:167–73.

10. Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther 2003;2:1093–103.

11. Akinaga S, Gomi K, Morimoto M, Tamaoki T, Okabe M. Antitumor activity of UCN-01, a selective inhibitor of protein kinase C, in murine and human tumor models. Cancer Res 1991;51:4888–92.

12. Mizuno K, Saido TC, Ohno S, Tamaoki T, Suzuki K. Staurosporine-related compounds, K252a and UCN-01, inhibit both cPKC and nPKC. FEBS Lett 1993;330:114–6.

13. Seynaeve CM, Kazanietz MG, Blumberg PM, Sausville EA, Worland PJ. Differential inhibition of protein kinase C isozymes by UCN-01, a staurosporine analogue. Mol Pharmacol 1994;45:1207–14.

14. Mizuno K, Noda K, Ueda Y, et al. UCN-01, an anti-tumor drug, is a selective inhibitor of the conventional PKC subfamily. FEBS Lett 1995;359:259–61.

15. Wang Q, Worland PJ, Clark JL, Carlson BA, Sausville EA. Apoptosis in 7-hydroxystaurosporine-treated T lymphoblasts correlates with activation of cyclin-dependent kinases 1 and 2. Cell Growth Differ 1995;6:927–36.

16. Akinaga S, Sugiyama K, Akiyama T. UCN-01 (7-hydroxystaurosporine) and other indolocarbazole compounds: a new generation of anti-cancer agents for the new century? Anticancer Drug Res 2000;15: 43–52.

17. Sausville EA, Lush RD, Headlee D, et al. Clinical pharmacology of UCN-01: initial observations and comparison to preclinical models. Cancer Chemother Pharmacol 1998;42(Suppl):S54–9.

18. Graves PR, Yu L, Schwarz JK, et al. The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. J Biol Chem 2000;275:5600–5.

19. Rundle NT, Xu L, Andersen RJ, Roberge M. G_2 DNA damage checkpoint inhibition and antimitotic activity of 13-hydroxy-15-oxozoapatlin. J Biol Chem 2001;276:48231–6.

20. Kohn EA, Yoo CJ, Eastman A. The protein kinase C inhibitor Go6976 is a potent inhibitor of DNA damage-induced S and G_2 cell cycle checkpoints. Cancer Res 2003;63:31–5.

21. Yu Q, La Rose J, Zhang H, Takemura H, Kohn KW, Pommier Y. UCN-01 inhibits p53 up-regulation and abrogates gamma-radiation-induced G_2-M checkpoint independently of p53 by targeting both of the checkpoint kinases, Chk2 and Chk1. Cancer Res 2002;62:5743–8.

22. Sato S, Fujita N, Tsuruo T. Interference with PDK1-Akt survival signaling pathway by UCN-01 (7-hydroxystaurosporine). Oncogene 2002;21:1727–38.

23. Alessi DR, James SR, Downes CP, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 1997;7:261–9.

24. Shaw M, Cohen P, Alessi DR. Further evidence that the inhibition of glycogen synthase kinase-3beta by IGF-1 is mediated by PDK1/PKB-induced phosphorylation of Ser-9 and not by dephosphorylation of Tyr-216. FEBS Lett 1997;416:307–11.

25. Chou TC, Talaly P. A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. J Biol Chem 1977;252:6438–42.

26. Alley MC, Pacula-Cox CM, Hursey ML, Rubinstein LR, Boyd MR. Morphometric and colorimetric analyses of human tumor cell line growth and drug sensitivity in soft agar culture. Cancer Res 1991;51: 1247–56.

27. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995;184:39–51.

28. Kim GS, Hong GS, Kim SW, et al. Leptin induces apoptosis via ERK/cPLA2/cytochrome C pathway in human bone marrow stromal cells. J Biol Chem 2003;278:21920–9.

29. Chou TC, Motzer RJ, Tong Y, Bosl GJ. Computerized quantitation of synergism and antagonism of Taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst (Bethesda) 1994;86:1517–24.

30. Chang TT, Gulati S, Chou TC, Colvin M, Clarkson B. Comparative cytotoxicity of various drug combinations for human leukemic cells and normal hematopoietic precursors. Cancer Res 1987;47:119–22.

31. Jimenez AI, Fernandez P, Dominguez O, Dopazo A, Sanchez-Cespedes M. Growth and molecular profile of lung cancer cells expressing ectopic LKB1: down-regulation of the phosphatidylinositol 3′-phosphate kinase/PTEN pathway. Cancer Res 2003;63:1382–8.

32. Rubin SJ, Hallahan DE, Ashman CR, et al. Two prostate carcinoma cell lines demonstrate abnormalities in tumor suppressor genes. J Surg Oncol 1991;46:31–6.

33. Carroll AG, Voeller HJ, Sugars L, Gelmann EP. p53 oncogene mutations in three human prostate cancer cell lines. Prostate 1993;23: 123–34.

34. McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. Cancer Res 1999;59:4291–6.

35. Zhao H, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol 2001;21:4129–39.

36. Fuse E, Tanii H, Kurata N, et al. Unpredicted clinical pharmacology of UCN-01 caused by specific binding to human alpha1-acid glycoprotein. Cancer Res 1998;58:3248–53.

37. Sausville EA, Arbuck SG, Messmann R, et al. Phase 1 trial of 72-hour continuous infusion UCN-01 in patients with refractory neoplasms. J Clin Oncol 2001;19:2319–33.

38. Monga RA, Messmann D, Headlee EW, et al. A Phase I trial of oral perifosine in patients with refractory neoplasm. Proc Am Soc Clin Oncol 2002;21:7b.

39. Hill DL, Tillery KF, Rose LM, Posey CF. Disposition in mice of 7-hydroxystaurosporine, a protein kinase inhibitor with antitumor activity. Cancer Chemother Pharmacol 1994;35:89–92.

40. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489–501.

41. Cantley LC. The phosphoinositide 3-kinase pathway. Science (Wash. DC) 2002;296:1655–7.

42. Mills GB, Lu Y, Fang X, et al. The role of genetic abnormalities of PTEN and the phosphatidylinositol 3-kinase pathway in breast and ovarian tumorigenesis, prognosis, and therapy. Semin Oncol 2001;28: 125–41.

43. Ruggeri BA, Huang L, Wood M, Cheng JQ, Testa JR. Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. Mol Carcinog 1998;21:81–6.

44. Cheng JQ, Ruggeri B, Klein WM, et al. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci USA 1996;93: 3636–41.

45. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15: 356–62.

46. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res 1998;58:2825–31.

47. Pegram M, Hsu S, Lewis G, et al. Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene 1999;18:2241–51.

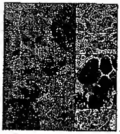
48. Pegram MD, Lopez A, Konecny G, Slamon DJ. Trastuzumab and chemotherapeutics: drug interactions and synergies. Semin Oncol 2000; 27:21–5; discussion 92–100.

49. Jia W, Yu C, Rahmani M, et al. Synergistic antileukemic interactions between 17-AAG and UCN-01 involve interruption of RAF/MEK- and AKT-related pathways. Blood 2003;102:1824–32.

50. Dai Y, Yu C, Singh V, et al. Pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) kinase/MAPK cascade interact synergistically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukemia cells. Cancer Res 2001;61:5106–15.

51. Yu C, Krystal G, Dent P, Grant S. Flavopiridol potentiates STI571-induced mitochondrial damage and apoptosis in BCR-ABL-positive human leukemia cells. Clin Cancer Res 2002;8:2976–84.

EXHIBIT 4



CLINICAL ONCOLOGY

Third Edition

MARTIN D. ABELOFF, MD
Eli Kennerly Marshall, Jr. Professor of Oncology
Professor of Medicine, Johns Hopkins University School of Medicine
Director, The Sidney Kimmel Comprehensive Cancer Center
at Johns Hopkins, Baltimore, MD

JAMES O. ARMITAGE, MD
Joe Shapiro Professor of Medicine, Department of Internal Medicine,
University of Nebraska Medical Center, Omaha, NE

JOHN E. NIEDERHUBER, MD
Professor, Departments of Surgery and Oncology, Wattawa Professor–
Bascom, University of Wisconsin–Madison, Madison, WI

MICHAEL B. KASTAN, MD, PHD
Chair, Department of Hematology/Oncology, St. Jude Children's
Research Hospital; Professor, Department of Pediatrics, University of
Tennessee School of Medicine, Memphis, TN

W. GILLIES MCKENNA, MD, PHD
Henry K. Pancoast Professor and Chair, Department of Radiation
Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA

ELSEVIER
CHURCHILL
LIVINGSTONE

ELSEVIER
CHURCHILL
LIVINGSTONE

The Curtis Center
170 S Independence Mall W 300E
Philadelphia, Pennsylvania 19106

CLINICAL ONCOLOGY ISBN 0-443-06629-9

Distributed in the United Kingdom by Churchill Livingstone, Robert Stevenson House, 1-3 Baxter's Place, Leith Walk, Edinburgh EH1 3AF, Scotland, and by associated companies, branches, and representatives throughout the world.

NOTICE

Medicine is an ever-changing field. Standard safety precautions must be followed, but as new research and clinical experience broaden our knowledge, changes in treatment and drug therapy may become necessary or appropriate. Readers are advised to check the most current product information provided by the manufacturer of each drug to be administered to verify the recommended dose, the method and duration of administration, and contraindications. It is the responsibility of the licensed prescriber, relying on experience and knowledge of the patient, to determine dosages and the best treatment for each individual patient. Neither the publisher nor the authors assume any liability for any injury and/or damage to persons or property arising from this publication.

Previous editions copyright 2000, 1995

International Standard Book Number 0-443-06629-9

Acquisitions Editor: Dolores Meloni
Developmental Editor: Donna Morrissey
Designer: Karen O'Keefe Owens
Executive Publisher: Susan F. Pioli

Printed in the United States of America.
Last digit is the print number: 9 8 7 6 5 4 3 2 1

46 Fever in the Neutropenic Cancer Patient

Alison G. Freifeld
Andre Kalil
Edward Rubenstein

SUMMARY OF KEY POINTS

INCIDENCE

- Neutropenia is a frequent occurrence in cancer patients due to the underlying disease or its therapy.
- The frequency of infectious complications is related to the degree and duration of neutropenia.
- Infection (either clinically or microbiologically defined) can be documented in about 40% of febrile episodes, and the remaining episodes are of unknown etiology. In either case, empirical antibiotic therapy is essential.

PATHOGENS

- The most common infecting organisms are gram-positive cocci, especially coagulase-negative staphylococci, viridans streptococci, and *Staphylococcus aureus*; the predominant gram-negative pathogens are *Escherichia coli*, *Klebsiella* spp., and *Pseudomonas aeruginosa*.
- Fungal infections are not uncommon in bone marrow transplant and leukemia patients and are usually caused by *Candida* spp. or *Aspergillus* spp. *Candida albicans* infections are infrequent in the setting of fluconazole prophylaxis, but non-*C. albicans* are increasing in incidence.

TREATMENT

- Cultures should be collected and antibiotic therapy should be instituted promptly.
- Antibiotic regimen should be active against the common gram-positive cocci and gram-negative bacilli (including *P. aeruginosa*) and may include monotherapy or combinations of antibiotics. However, vancomycin should be strictly reserved for specific indications.
- Low-risk patients (no medical comorbidities) may be treated as outpatients with oral ciprofloxacin plus amoxicillin-clavulanate.
- Predominant pathogens within the hospital and their antibiotic-susceptibility patterns should influence antibiotic selection.
- If the patient has persistent fever after 3 to 4 days, and the infecting organism has not been identified, therapy may be modified if the patient is unstable or new clinical or microbiologic data dictate a change.
- Often it is necessary to institute antifungal therapy on an empirical basis after day 5 to 7 of broad-spectrum antibiotics if the patient is still febrile.
- Treatment of fungal infections is seldom successful unless the neutropenia resolves.
- Colony-stimulating factors and white blood cell transfusions may be considered in some neutropenic patients with a documented infection who are not responding to antimicrobial therapy.

INTRODUCTION

Neutropenia is a common and predictable consequence of many cytotoxic cancer therapies and a frequent complication of malignancies that impair bone marrow function. Neutrophilic granulocytes are a critical component of host defenses, primarily against bacterial and fungal pathogens. They mediate many inflammatory responses toward invading organisms to contain and eliminate infections. Accordingly, a deficit of neutrophils (i.e., neutropenia) is associated with an increase in susceptibility to infections as well as an attenuation of inflammatory responses to infections. Clinical signs and symptoms of inflammation may be muted, even in the setting of active infection in the neutropenic patient. Infection unopposed by innate neutrophil responses can progress rapidly and relentlessly, leading to high levels of morbidity and mortality. Oncologists must be aware of this risk and approach neutropenic cancer patients with care and vigilance.[1]

This chapter focuses on infections during the early phases of chemotherapy-induced neutropenia, primarily in relatively lower risk patients (i.e., those who have solid tumors or who are undergoing autologous stem cell transplant). Patients with acute leukemia or those undergoing allogeneic stem cell transplant are at higher risk for serious infections, and the spectrum of infections in those patients is expanded, as described in Chapter 47.

NEUTROPENIA AS A RISK FACTOR FOR INFECTION

page 925
page 926

Add to lightbox

Figure 46-1 Relation between neutrophil count and infection in patients with acute leukemia.

The association between neutropenia and increased infection risk was initially demonstrated by Bodey and colleagues[2] in 1966 in a study of leukemic patients undergoing cytotoxic therapy. The data show that the frequency of infectious complications is inversely related to the degree and duration of neutropenia (Fig. 46-1). Infection risk starts to increase when the neutrophil count decreases to less than 1000 cells/mm^3 and increases dramatically when it is less than 500 cells/mm^3. Fewer than half of the neutropenic patients who become febrile will have an identified or occult infection. In roughly 10% to 20% or more of patients with neutrophil counts less than 100 cells/mm^3, a bloodstream infection will develop. The remainder of patients with fever and neutropenia have a "fever of undetermined origin" (FUO), with no identifiable source despite examination and cultures.[1-3]

The duration of neutropenia also is an important determinant of both infection risk and infection type. Brief durations of neutropenia, particularly those lasting less than 7 days, are associated with a rapid and favorable response to empirical antibiotic therapy.[4] A neutrophil count persistently less than 500 cells/mm^3 for more than 10 days is considered to represent a "high risk" state. Such patients are not only at high risk of developing an infection, but they also are at greater risk for infection-related morbidity and mortality as a consequence of prolonged neutropenia. Prolonged and profound neutropenia is a particular risk for acquiring invasive fungal disease such as *Aspergillus*, a frequently fatal invasive mold infection. The pathogens responsible for initial infections, early in the course of fever and neutropenia, are primarily bacteria and viruses, whereas antibiotic-resistant bacteria, yeast, fungi, and viruses are common causes of subsequent infections. Deaths are usually due to these subsequent infections. Mortality due to initial infections is relatively rare.[5,6]

In addition to cytotoxic chemotherapy, other cases of neutrophil deficiency may be due to bone marrow incompetence as a result of myelodysplastic syndrome or to crowding out of normal granulocytic precursors by tumor cells. "Functional neutropenia" due to impaired neutrophil microbicidal activity may arise as a consequence of underlying disease such as leukemia or therapies such as steroids. Ineffective neutrophil killing leaves the patient highly vulnerable to infection despite seemingly normal peripheral white blood cell counts.

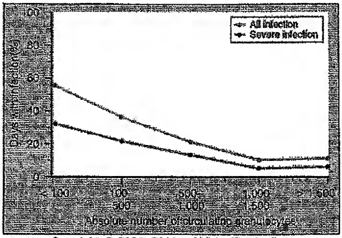


Figure 46-1 Relation between neutrophil count and infection in patients with acute leukemia.

X. RELATED PROCEEDINGS APPENDIX

None